Supporting Information

Altered cofactor regulation with disease associated p97/VCP mutations

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Classification: Biological Science
Biochemistry

SI Methods

BIOMOL Green ATPase assay — Purified p97 (25 μL of 50 μM; final concentration in the reaction was 25 nM monomer concentration) was diluted in 30 mL of assay buffer [10 mL of 5 x assay buffer A (1 x = 50 mM Tris pH 7.4, 20 mM $MgCl_2$, 1 mM EDTA,) mixed with 20 mL water and 50 μL 0.5 M TCEP, 50 μL 10% Triton] to make the enzyme solution. 30 μL of the enzyme solution was dispensed into each well of a 96-well plate and 10 μL of p97 cofactor (0 – 4000 nM) was added into each well. The ATPase assay was carried out by adding 10 μL of 1000 μM or 4000 μM ATP (pH 7.5) to each well and incubating the reaction at room temperature for 35 min. Reactions were stopped by adding 50 μL of BIOMOL Green reagent (Enzo Life Sciences). Absorbance at 635 nm was measured after 4 min. Eight final ATP concentrations were used to determine steady-state kinetic constants. For mutants, reaction times were adjusted according to specific activities in order to obtain acceptable absorbance readings. Michaelis-Menten constants were calculated from 8 replicates by fitting data to Equation 1 using GraphPad Prism 6.0.

$$v = V_{max}*[ATP]/(K_m + [ATP]) \qquad (1)$$

In vitro His pull-down assays **—**12 μM His-tagged p97 was mixed with 30 μM p47, p37, or p47 variants in 100 μL buffer [20 mM HEPES, pH 7.4, 250 mM KCl, 1mM $MgCl_2$, 200 μM ATP, 0.01% Triton, 10 μL MagneHis™ Ni-Particles (Promega, V8560)] at room temperature for 30 min. Unbound supernatant was removed, and Ni-Particles were washed with 500 μL washing buffer (50 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 20 mM imidazole) 3 times. Bound proteins were eluted by adding 100 μL 1x Laemmli Sample Buffer (Bio-Rad) and heated to 90^{o}C for 5 min. Samples were separated on 4-20% Tris-Glycine Gel (Bio-Rad) and stained with Coomassie blue (Thermo Scientific Imperial Protein Stain).

Microscale Thermophoresis (MST) — MST was carried out at 25 °C on a Monolith NT.115pico instrument (Nano-Temper Technologies). Full-length p37 and p47 were exchanged to 1x assay Buffer A containing TCEP and Pluronic acid (50 mM Tris pH 7.4, 20 mM $MgCl_2$, 1 mM EDTA, 0.5 mM TCEP, 0.05% Pluronic acid) and labeled with NanoTemper 647 cysteine-reactive red dye per the manufacturer's instructions. In this assay, unlabeled p37 or p47 was titrated against 400 pM of NT-647 labeled p37 or p47 in two fold steps from 250 nM to 61 pM. Assays were performed in hydrophilic capillaries in two separate experiments.

Surface plasmon resonance (SPR) **—** Binding affinities for p37 and p47 cofactors to p97 proteins were measured on a Biacore 4000 instrument. NeutrAvidin-coated sensor chips were prepared as described (1) with the following exceptions: 60 mM *N*-hydroxysuccinimide and 240 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide was injected for 4 min, followed by a 2 min injection of 0.25 μg/mL NeutrAvidin. p97 proteins were immobilized in 10 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mM TCEP, 0.05% Tween 20 to 500-600 RU by injecting 4-6 μg/mL protein for 2 min.

Binding of cofactor proteins was measured in 25 mM Tris, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 0.5 mM TCEP, 0.05% Tween 20 and 0.1% Prionex (Calbiochem) at 20 °C. Sensorgrams were reduced, double-referenced, and fit to a 1:1 kinetic interaction model (p37) or to an equilibrium binding model for a 1:1 interaction (F253S p47) or a 2:1 interaction (WT p47, Δ69-92 p47, Δ83-92 p47 and 83-88 ($DE_5$to NQ_5) p47) in Scrubber 2 (BioLogic Software).

p97-mediated post-mitotic Golgi reassembly assay — The assay was carried out *in vitro* with purified Golgi membranes, p97, and its cofactors (2,3). Briefly, purified rat liver Golgi stacks were treated with mitotic cytosol, and the resulting membrane fragments were incubated with indicated amounts of purified p97/p47, p97/Δ69-92 p47, or p97/p37 proteins for reassembly. Membranes were processed for EM, and the results were quantified to estimate the activity of membrane fusion to form cisternal membranes. Mitotic Golgi fragments were normalized to 0%. Reassembly with WT p97 (167 nM hexamer) and p47 (167 nM trimer) was normalized to 100%. The results represent the mean of at least ten EM images ± SEM.

Western blot analysis — Human osteosarcoma U2OS cells were maintained in DMEM supplemented with penicillin/streptomycin and 10% fetal bovine serum. Cells were 80% confluent at the time of transfection with Flag-p47 plasmid. Transfections were performed with BioT reagent (Bioland Scientific LLC). Cells were lysed with Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). The sample (25 μg) was loaded on a 4-20% gel for SDS-PAGE (Bio-Rad). Proteins were transferred to nitrocellulose membranes using the Trans-Blot Turbo system (Bio-Rad). Antibodies used include p97 (MA3-004, Thermo Scientific), Flag (F3165, Sigma-Aldrich), p62 (M162-3, MBL International Corporation), p47 (15620-1-AP, Proteintech Group Inc.), LC3 (PM036, MBL International Corporation), and GAPDH (2118, Cell Signaling Technology).

Determining IC_{50} values of p97 inhibitors in ATPase assays — The detailed method was described previously (1,4). Inhibition of human p97 (25 nM monomer) was carried out in assay buffer (50 mM Tris pH 7.4, 20 mM $MgCl_2$, 1 mM EDTA, 0.5 mM TCEP) containing 0.01% Triton X-100 and 200 μM ATP. The 8-dose titration was performed at 40, 13.3, 4.4, 1.48, 0.49, 0.16, 0.05, and 0 μM. ATPase activity was determined through the addition of Biomol Green Reagent (Enzo Life Sciences). p47 was added to a final concentration of 400 nM. NMS-873 was purchased from Xcess Biosciences Inc.

Supplemental Tables.

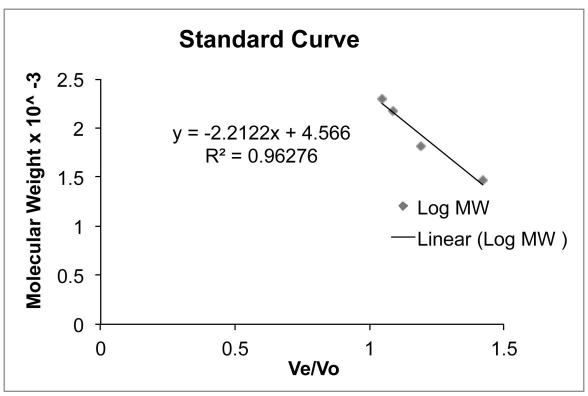
Table S1. Plasmids used in this study

Plasmid Number	Plasmid name	Vector	Source and Reference
TCB-197/ SLB-001	Human p97 pET15_T	pET15b_TEV linker	Chou, 2014*
TCB-213	E305Q human p97 pET15_T	pET15b_TEV linker	Chou, 2014*
TCB-221	E578Q human p97 pET15_T	pET15b_TEV linker	Chou, 2014*
TCB-210	R155H human p97 pET15_T	pET15b_TEV linker	This study
TCB-366	L198W human p97 pET15_T	pET15b_TEV linker	This study
TCB-211	A232E human p97 pET15_T	pET15b_TEV linker	This study
TCB-256	A232E, E305Q human p97 pET15_T	pET15b_TEV linker	This study
TCB-249	A232E, E578Q human p97 pET15_T	pET15b_TEV linker	This study
TCB-403	R155H, E305Q human p97 pET15_T	pET15b_TEV linker	This study
TCB-404	R155H, E578Q human p97 pET15_T	pET15b_TEV linker	This study
TCB-328	Rat p47 pET15_T	pET15b_TEV linker	Chou, 2014*
TCB-424	Δ69-92 rat p47 pET15_T	pET15b_TEV linker	This study
TCB-431	Δ83-90 rat p47 pET15_T	pET15b_TEV linker	This study
TCB-432	83-88 ($DE_5$to NQ_5) rat p47 pET15_T	pET15b_TEV linker	This study
TCB-377	F253S rat p47 pET15_T	pET15b_TEV linker	This study
TCB-327	Human p37 pET15_T	pET15b_TEV linker	This study
TCB-451	Flag-rat p47	pCMV5B	Alexandru, 2008**
SLB-024	C-AviTag human p97 pET15_T	pET15b_TEV linker	Chou, 2014*
SLB-049	R155H C-AviTag human p97 pET15_T	pET15b_TEV linker	This study
SLB-055	A232E C-AviTag human p97 pET15_T	pET15b_TEV linker	This study
---	pBirAcm	pACYC184	Avidity

*Chou, T. F., Bulfer, S. L., Weihl, C. C., Li, K., Lis, L. G., Walters, M. A., Schoenen, F. J., Lin, H. J., Deshaies, R. J., and Arkin, M. R. (2014) Specific inhibition of p97/VCP ATPase and kinetic analysis demonstrate interaction between D1 and D2 ATPase domains. *J. Mol. Biol.* **426**, 2886-2899.
** Alexandru, G., Graumann, J, Smith, G. T., Kolawa, N. J., Fang, R., and Deshaies, R. J. (2008) UBXD7 binds multiple ubiquitin ligases and implicates p97 kin HIF1alpha turnover. Cell **134**, 804-816.

Table S2. Gel filtration to determine the oligomeric states of p37, p47, and p47 variants. Gel filtration was carried out with Superdex 75 10/300 GL (GE Healthcare). The column was calibrated with molecular weight (MW) standards kit (Sigma). The standards are Blue Dextran [MW is 2000 kDa; used to determine the void volume (Vo) of the column], β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). The linear molecular weight calibration plotted Ve/Vo versus Log MW (see standard curve), where Ve is the elution volume of the MW standard and Vo is the void volume. To determine the apparent molecular weight of p37, p47, or p47 variants, we injected 100 μL of 20 μM proteins, determined their elution volumes (Ve), and then calculated apparent molecular weights using the equation obtained from the standard curve. The calculated MW was divided by the monomer MW to calculate the oligomeric state.

Molecular Weight Standard	Molecular Weight (kDa)	Elution Volume (mL)	Ve/Vo	Log MW
Carbonic Anhydrase	29	12.15	1.421	1.462
Bovine Serum Albumin	66	10.2	1.192	1.819
Alcohol Dehydrogenase	150	9.3	1.087	2.176
β-Amylase	200	8.95	1.046	2.301
Blue Dextran	2000	8.55	1	



Proteins	**Ve (mL)**	**Ve/Vo**	**Log (calculated MW)**	**Calculated MW (kDa)**	**Monomer MW (kDa)**	**Oligomer***
WT p47	9.8	1.15	2.03	107.3	40.87	2.6
WT p37	9.9	1.16	2.00	101.0	37.27	2.7
83-88 (DE_5 to NQ_5) p47	9.8	1.15	2.03	107.3	40.87	2.6
Δ83-90 p47	9.9	1.16	2.00	101.0	39.88	2.5
47-371 p47 (ΔUBA p47)	9.9	1.16	2.00	101.0	36.18	2.8
Δ69-92 p47	10.1	1.18	1.95	89.7	37.97	2.4

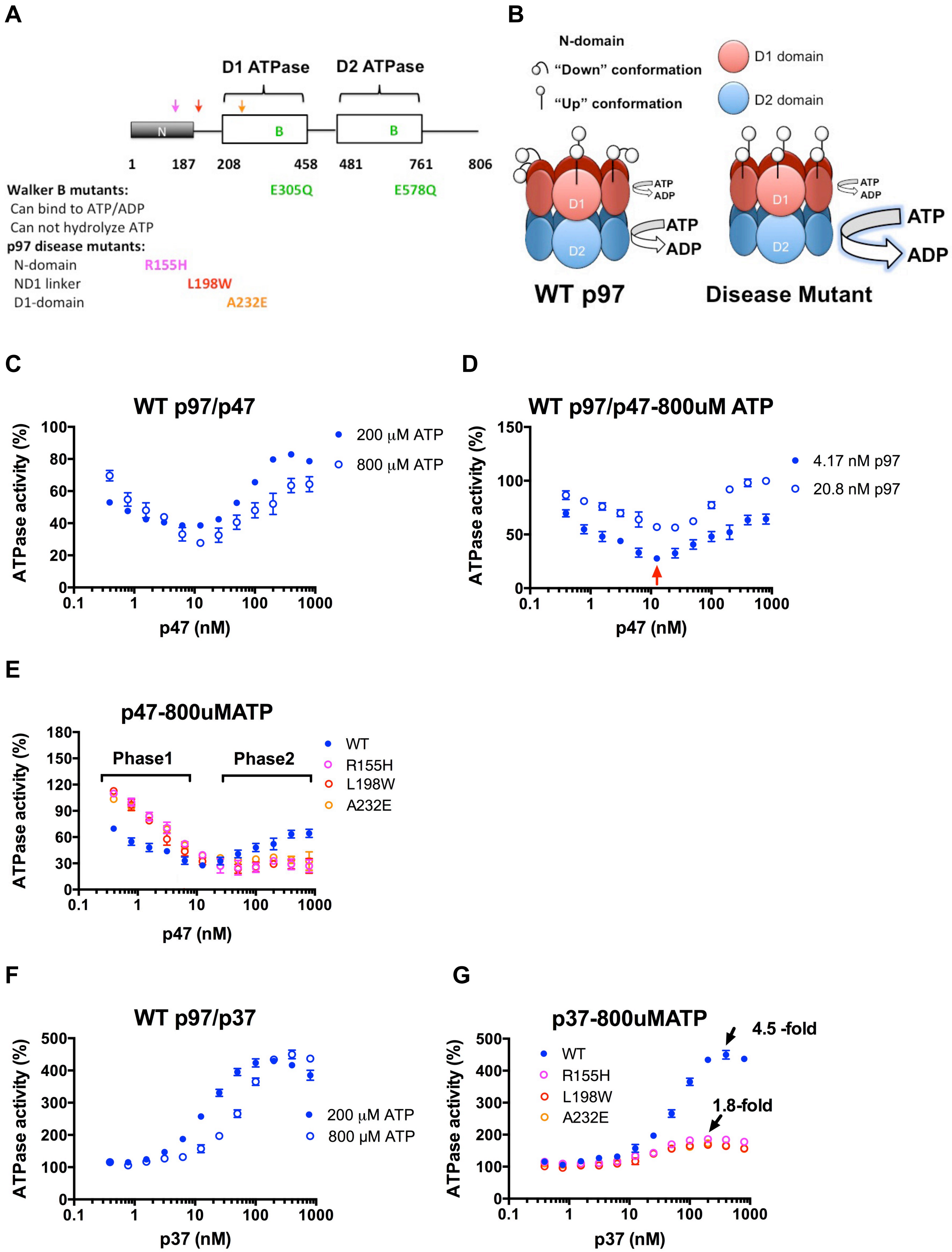
*** The calculated MW was divided by the monomer MW to calculate the oligomer number.**

Table S3. Binding affinities for p97 cofactors to WT p97 and disease mutants R155H and A232E, measured by SPR.

	WT p97		R155H p97		A232E p97	
	K_{D1} μM	K_{D2} μM	K_{D1} μM	K_{D2} μM	K_{D1} μM	K_{D2} μM
WT p37[1]	0.016		0.015		0.015	
WT p47[2]	0.026	1.4	0.021	0.098	0.019	0.70
F253S p47[3]	5.6		2.93		2.36	
Δ69-92 p47[2]	0.023	1.5	0.018	1.1	0.016	0.71
Δ83-90 p47[2]	0.030	1.2	0.023	1.0	0.021	0.91
83-88 (DE_5 to NQ_5) p47[2]	0.032	1.4	0.025	0.87	0.022	0.70

[1]K_D values determined using a one-site kinetic fit.
[2]K_D values determined using a two-site equilibrium fit.
[3]K_D values determined using a one-site equilibrium fit.

Supplemental Figure
Figure S1



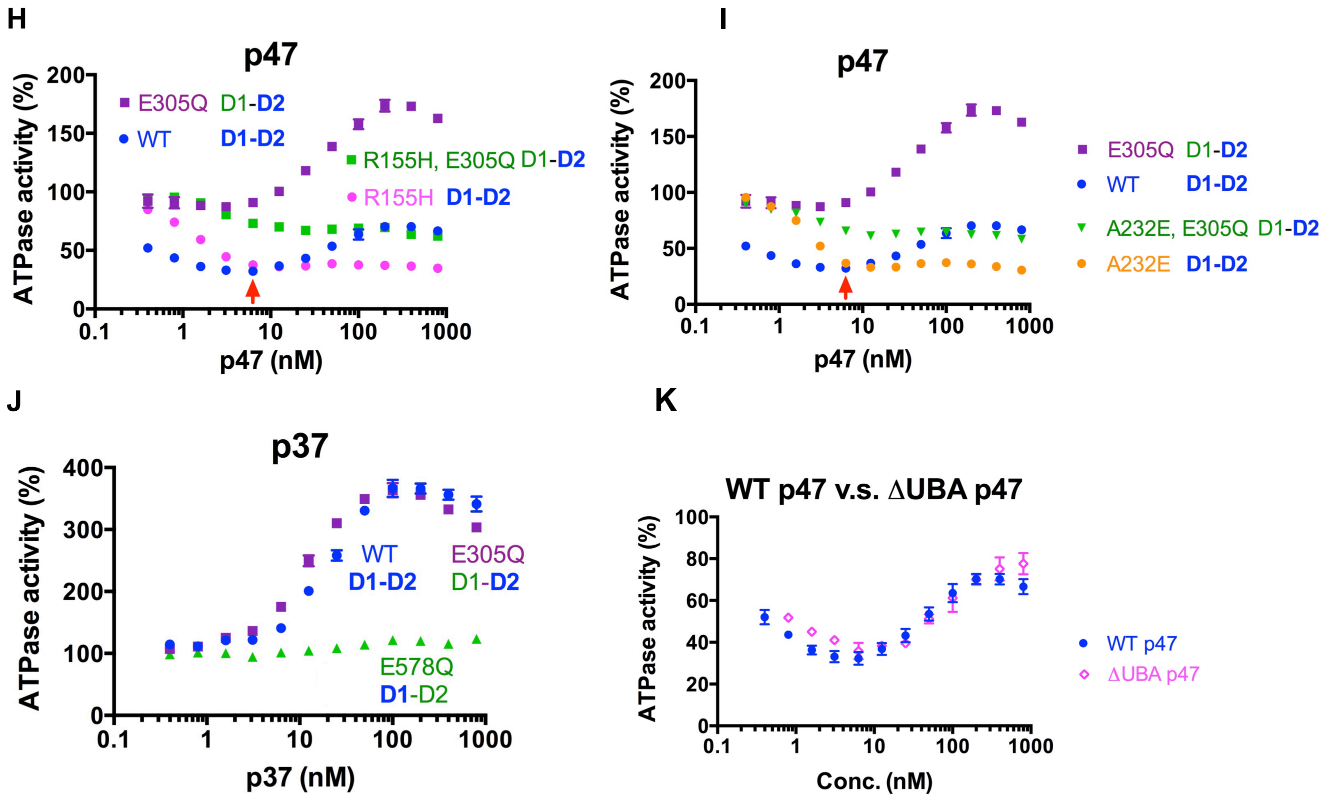


Figure S1. **Regulation of p97 ATPase activity by p37 and p47.** (A) Diagram showing the domain representation of human p97 and the mutations analyzed in this study. (B) Differences in N-domain conformations between WT and p97 disease mutants. (C) Normalized WT ATPase activities (4.17 nM hexamer) in the presence of increasing amounts of p47 (0 to 800 nM) for 200 μM versus 800 μM ATP. Error bars indicate ± SD (n=6). (D) Normalized WT ATPase activities (4.17 nM versus 20.8 nM hexamer) in the presence of increasing amounts of p47 (0 to 800 nM) for 800 μM ATP. Error bars indicate ± SD (n=6). (E) Normalized ATPase activities of WT, R155H, L198W, and A232E p97 (4.17 nM hexamer) in the presence of increasing amounts of p47 (0 to 800 nM) with 800 μM ATP. Error bars indicate ± SD (n=12, excluding L198W, where n=6). (F) Normalized WT ATPase activities (4.17 nM hexamer) in the presence of increasing amounts of p37 (0 to 800 nM) for 200 μM versus 800 μM ATP. Error bars indicate ± SD (n=6). (G) Normalized ATPase activities of WT, R155H, L198W, and A232E p97 (4.17nM hexamer) in the presence of increasing amounts of p37 (0 to 800 nM) with 800 μM ATP. Error bars indicate ± SD (n=6). (H) ATPase activities of WT, D1-E305Q, R155H, and the R155H, D1-E305Q double mutant were measured in the presence of p47 (0 to 800 nM) with 200 μM ATP. Error bars indicate ± SD (n=12, excluding double mutant R155H,E305Q, where n=6). (I) Normalized ATPase activities of WT, D1-E305Q, A232E, and the A232E, D1-E305Q double mutant were measured in the presence of p47 (0 to 800 nM). Error bars indicate ± SD (n=12). (J) ATPase activities of D1-E305Q and D2-E578Q were measured in the presence of p37 with 200 μM ATP. Error bars indicate ± SD (n=6). (K) Normalized ATPase activities of WT (4.17 nM hexamer) in the presence of increasing amounts of WT p47 and ΔUBA p47 (0 to 800 nM) with 200 μM ATP. The activity of each p97 protein was normalized to its basal activity in the absence of cofactor. Blue lettering indicates the active ATPase domain in each protein, and green lettering indicates the Walker B mutant. Error bars indicate ± SD (n=12 for WT p47, n=6 for ΔUBA p47).

Figure S2

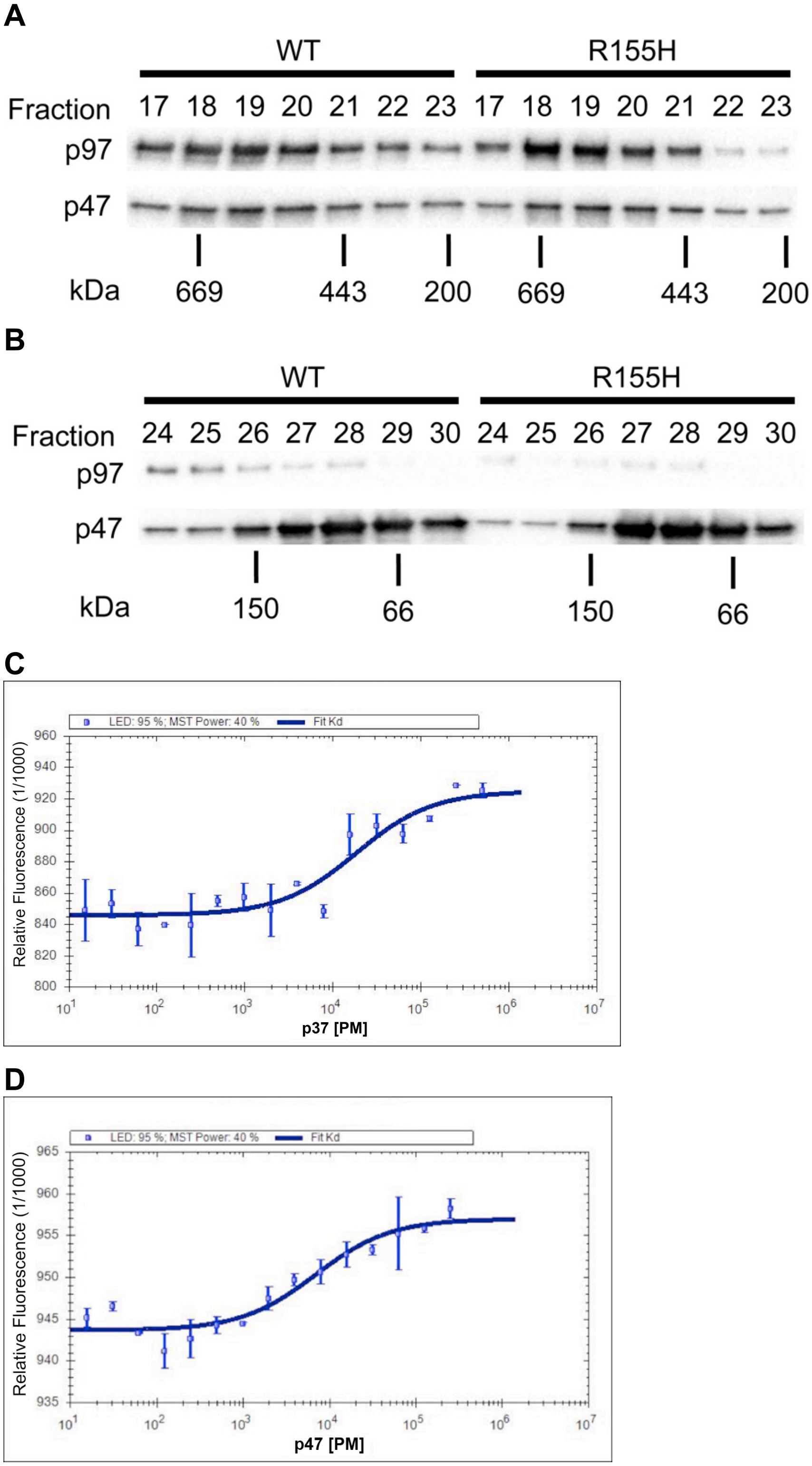


Figure S2. Gel filtration and microscale thermophoresis analysis. Gel filtration was used to determine the stability of the p97-p47 complex for both WT and R155H p97. 1.67 μM p97 hexamer was mixed with 80 μM p47 for 10 min and fractionated in HEPES buffer (20 mM HEPES, 150 mM KCl, 1 mM $MgCl_2$, 5% glycerol, pH 7.4) with a gel filtration column (Superdex 20010/300 GL, GE Healthcare). (A) Western blot for Fractions 17 to 23. (B) Western blot for Fractions 24 to 30. Microscale thermophoresis (MST) analysis was used to determine the equilibrium binding constant

(K_D) for trimer formation in solution for both (C) p37 and (D) p47. Unlabeled p97 cofactor was titrated against 400 pM of NT-647 labeled cofactor in two fold steps from 500,000 pM to 15 pM. Assays were performed in hydrophilic capillaries using a Monolith NT.115pico instrument.

Figure S3

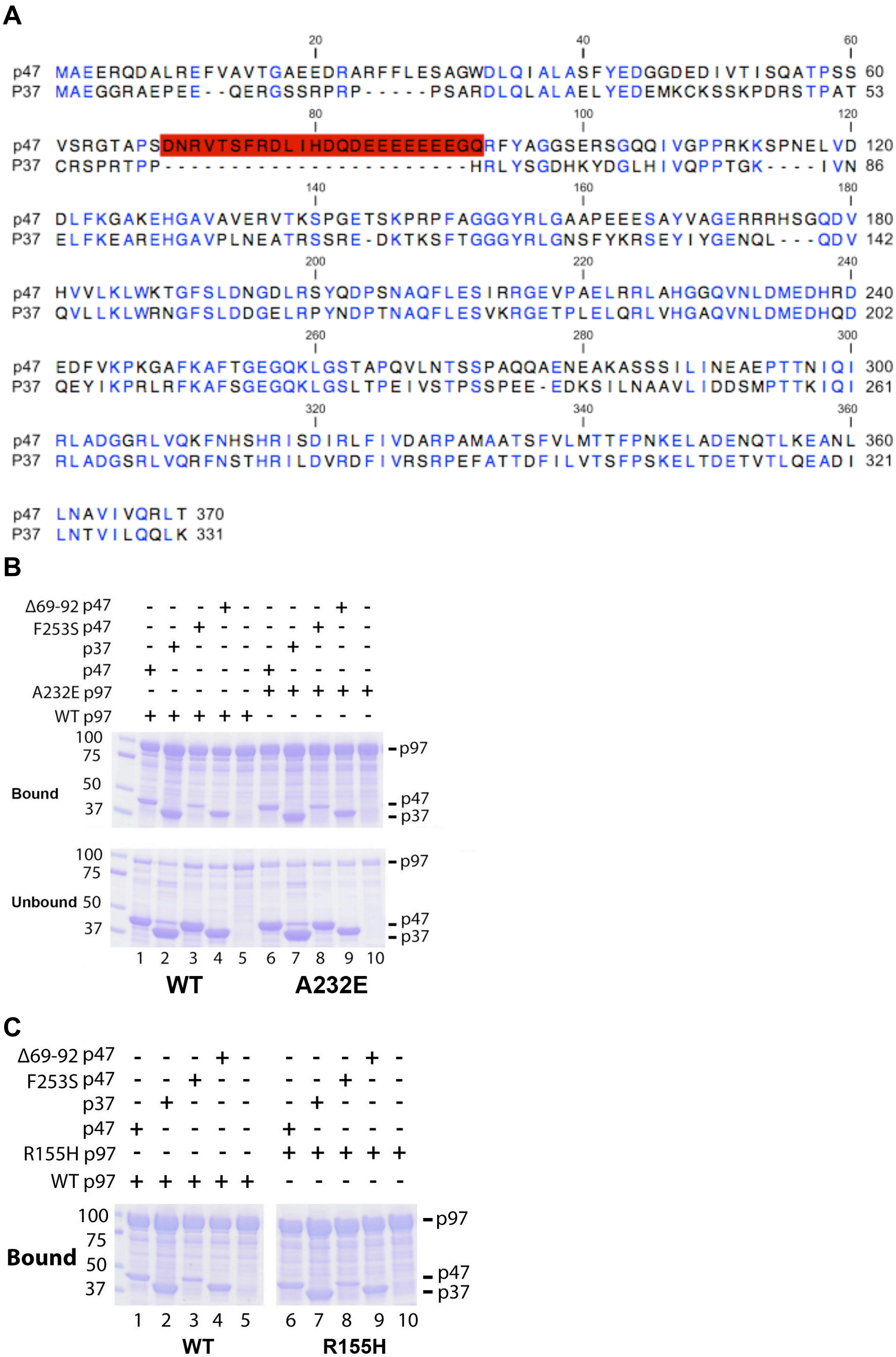


Figure S3. WT p97 and disease mutants display similar binding to p47, and removal of p47 amino acids 69-92 does not affect p47-p97 interactions. (A) p47 and p37 sequence alignment.

Amino acid sequences were aligned using CLC Sequence Viewer 6 (CLC Bio). The alignment showed that p47 amino acid residues 69-92 (in *red*) are completely absent in p37. (B) and (C) An *in vitro* His pull-down assay was carried out by mixing 12 μM His-tagged p97 with 30 μM p47, p37, F253S p47, or Δ69-92 p47 at room temperature for 30 min. Unbound supernatant was removed, and Ni-Particles were washed with 500 μL washing buffer (50 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 20 mM imidazole) 3 times. Bound proteins were eluted by adding 100 μL 1x Laemmli Sample Buffer (Bio-Rad) and heated at 90^{o}C for 5 min. Samples were separated on 4-20% Tris-Glycine Gels (Bio-Rad) and stained with Coomassie blue (Thermo Scientific Imperial Protein Stain). (B) Comparison of WT and A232E p97. (C) Comparison of WT and R155H p97.

Figure S4

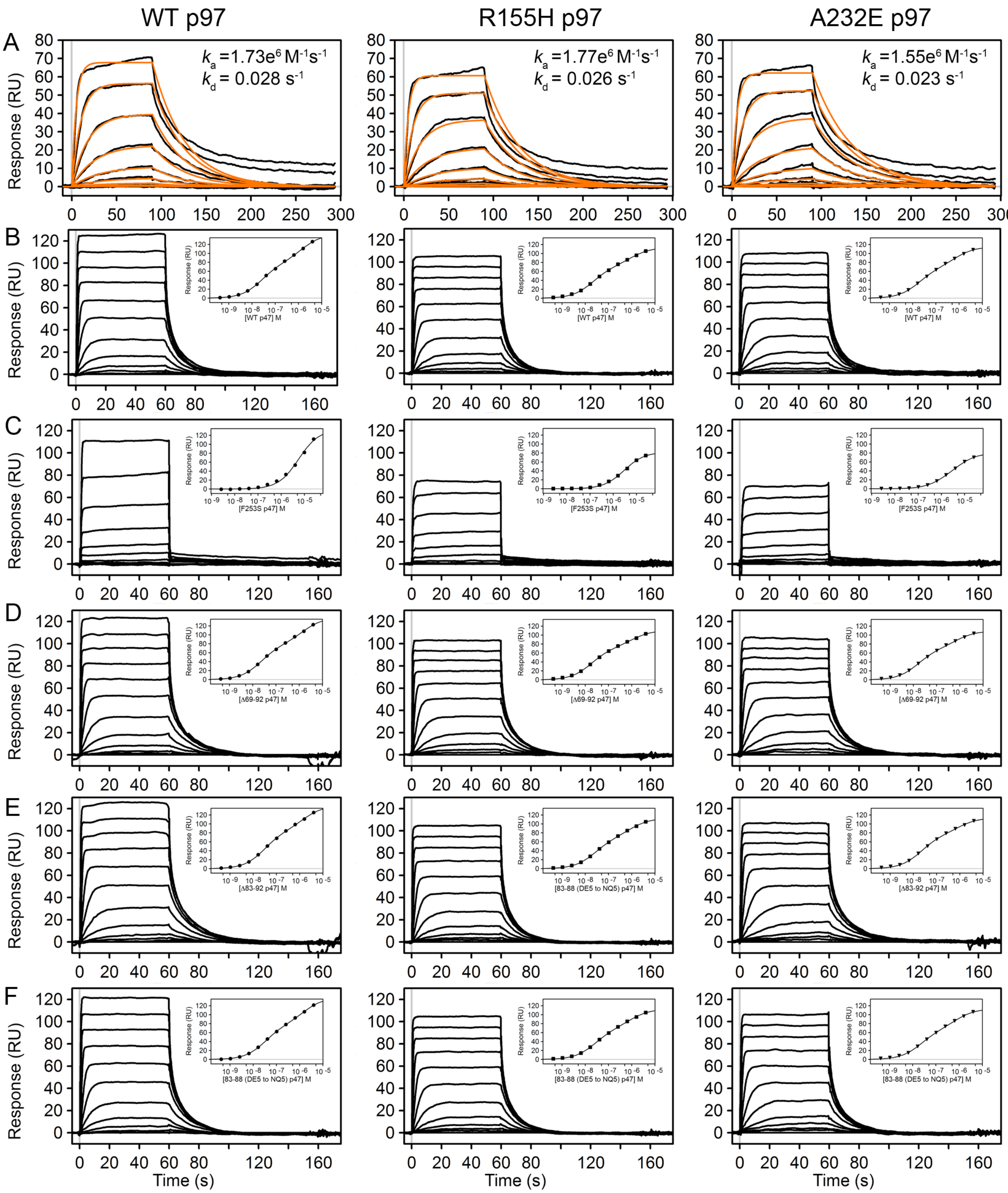
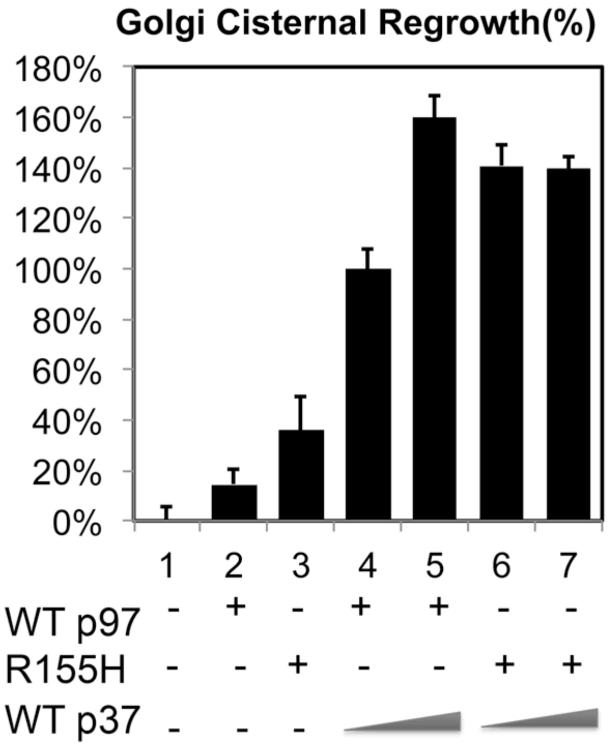


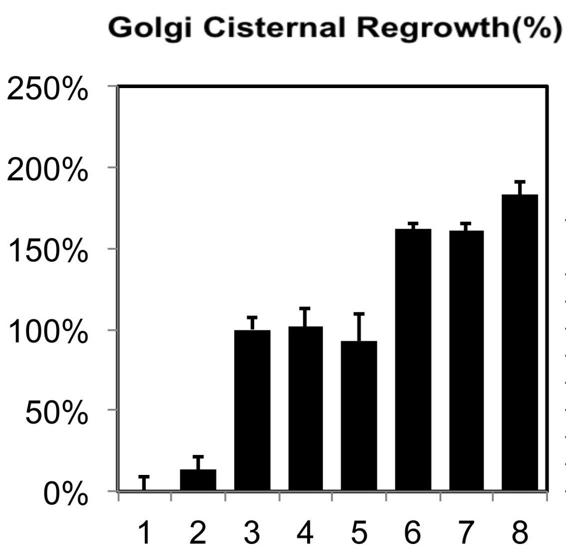
Figure S4. SPR sensorgrams for p37 and p47 binding to WT p97 and disease mutants R155H and A232E. (A) Binding of WT p37 (67 pM-102.4 nM; 2.5 fold dilutions) to immobilized p97 fit to a 1:1 kinetic model (orange line). (B) Binding of WT p47 (419 pM-4 μM; 2.5 fold dilutions) to p97 fit to a two-site equilibrium-binding model (inset). (C) Binding of F253S p47 (2.6 nM-25 μM; 2.5 fold dilutions) to p97 fit to a one-site equilibrium-binding model (inset). (D-F) Binding of Δ69-92 p47 (D) Δ83-92 p47 (E) and 83-88 DE5 to NQ5 (F) (419 pM-4 μM; 2.5 fold dilutions) to p97 fit to a two-site equilibrium-binding model (insets).

Figure S5

A



B

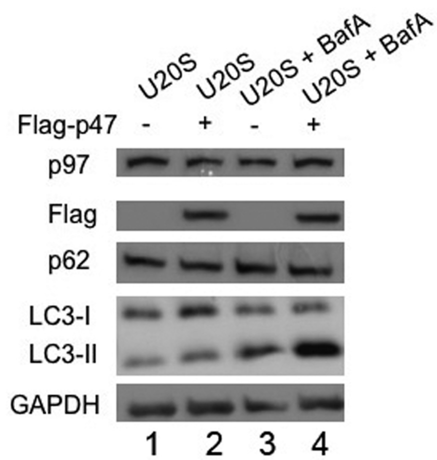


column #	p97 Type	p97 Concentration	p47 or p37 Type	p47 or p37 Concentration
1	–	–	–	–
2	WT	167 nM hexamer	–	–
3	WT	167 nM hexamer	WT p47	167 nM trimer
4	WT	167 nM hexamer	WT p47	1336 nM trimer
5	WT	167 nM hexamer	Δ69-92 p47	167 nM trimer
6	WT	167 nM hexamer	Δ69-92 p47	1336 nM trimer
7	WT	167 nM hexamer	WT p37	167 nM trimer
8	WT	167 nM hexamer	WT p37	1336 nM trimer

Figure S5. The R155H disease mutant displays elevated activity and reduced responses to the activating effect of p37, as shown by a p97-mediated post-mitotic Golgi reassembly assay. The assay was carried out *in vitro* with p97 cofactors. Purified rat liver Golgi stacks were treated with mitotic cytosol, and the resulting membrane fragments were re-isolated by centrifugation and further incubated with purified p97 and either p47, Δ69-92 p47, or p37. Membranes were processed for EM, and the results were quantified to estimate the percentage of cisternal membranes fro the activity of cisternal membrane regrowth. Mitotic Golgi fragments were normalized to 0%. Reassembly with p97 (167 nM hexamer) and p47 (167 nM trimer) was normalized to 100%. The results represent the mean of at least ten EM images ± SEM. (A) p97-mediated post-mitotic Golgi reassembly assays. Column 1: Control (mitotic Golgi fragments), 2: WT p97 alone (167 nM hexamer), 3: R155H p97 alone (167 nM hexamer), 4: WT p97 (167 nM hexamer) with 167 nM p37 trimer, 5: WT p97 (167 nM) with 1336 nM p37 trimer, 6: R155H p97 (167 nM) with 167 nM p37 trimer, 7: R155H p97 (167 nM) with 1336 nM p37 trimer. (B) Column 1: Control, 2: WT p97 alone (167 nM hexamer), 3: WT p97 (167 nM hexamer) with 167 nM p47 trimer, 4: WT p97 (167 nM hexamer) with 1336 nM p47 trimer, 5: WT p97 (167 nM hexamer) with 167 nM Δ69-92 p47 trimer, 6: WT p97 (167 nM) with 1336nM Δ69-92 p47 trimer, 7: WT p97 (167 nM) with 167 nM p37, 8: WT p97 (167 nM) with 1336 nM p37.

Figure S6

A



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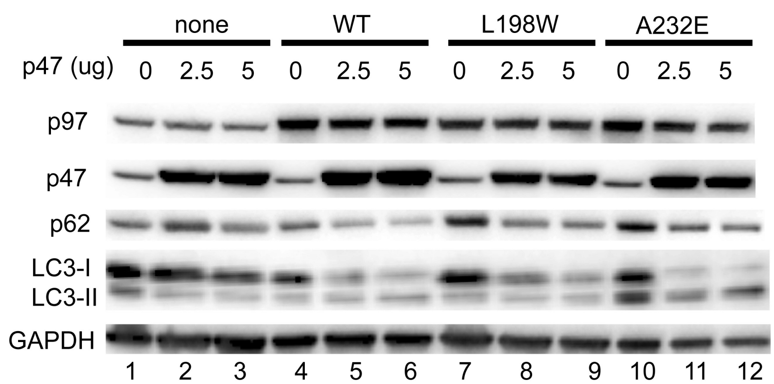

Figure S6. p47 improves p97/VCP disease associated autophagy impairment. (A) Western blot assays to evaluate the effect of p47 in the autophagy pathway. U2OS cells were transiently transfected with Flag-p47 plasmid for 48 h to overexpress Flag-p47 (lanes 2 and 4). After 44 h of transfection, U2OS cells were treated with 200 nM Bafilomycin A for 4 h, to perform an "autophagic flux" assay (lane 3 and 4). GAPDH served as a loading control. (B) Western blot assays to evaluate the effects of p47 on the disease mutant in the autophagy pathway. U2OS cells stably expressing WT, L198W, or A232E p97 proteins were used to determine the effect of p47 on the autophagy pathway. Cells were transiently transfected with Flag-p47 plasmid (2.5 or 5 µg) for 30 h. GAPDH served as a loading control.

Figure S7
A

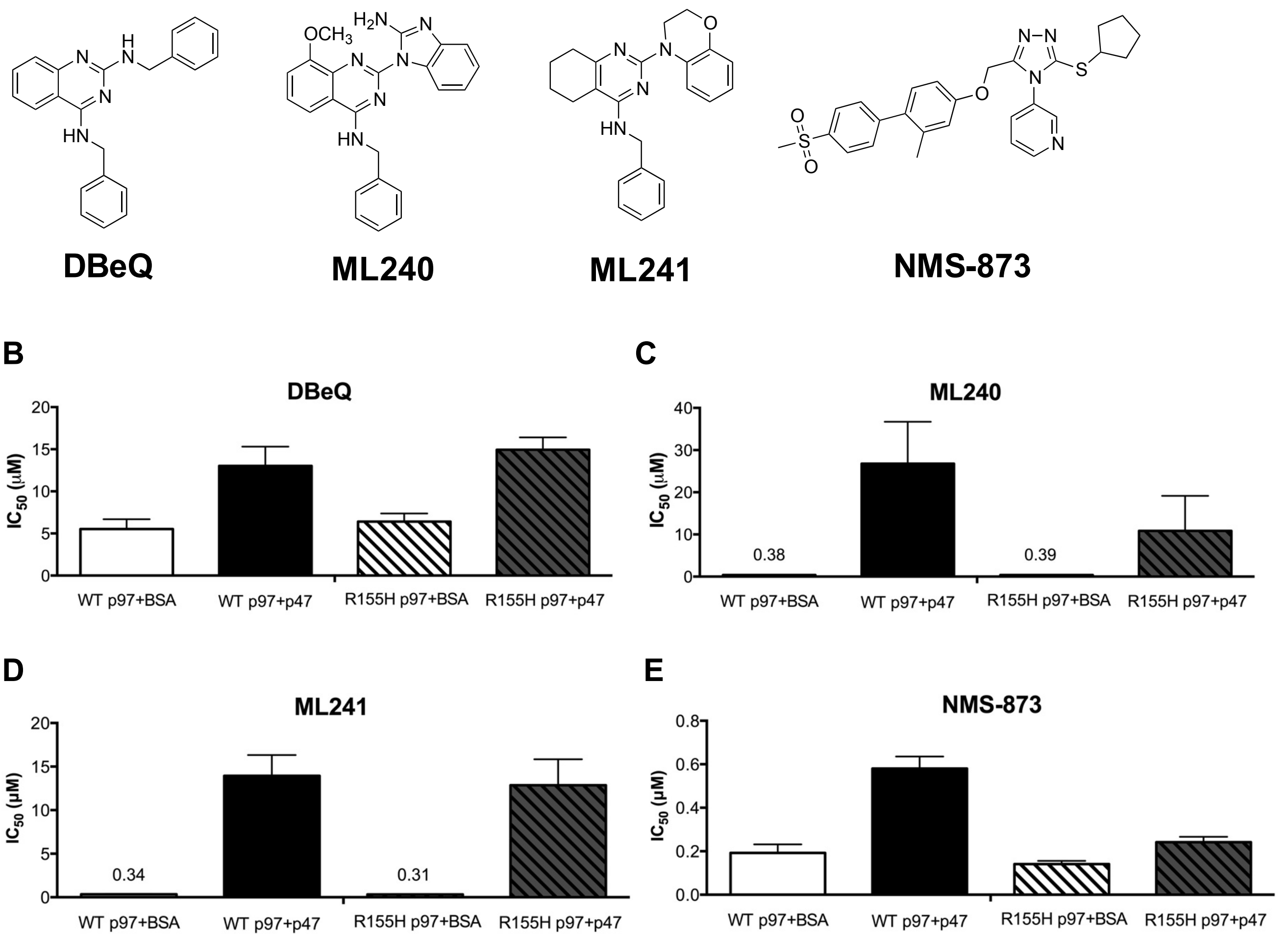

Figure S7. IC_{50} values of p97 inhibitors against WT p97, the p97-p47 complex, R155H p97, and the R155H p97-p47 complex. (A) Chemical structures of DBeQ, ML240, ML241, and NMS-873. (B) IC_{50} values of DBeQ. (C) IC_{50} values of ML240. (D) IC_{50} values of ML241. (E) IC_{50} values of NMS-873.

Figure S8

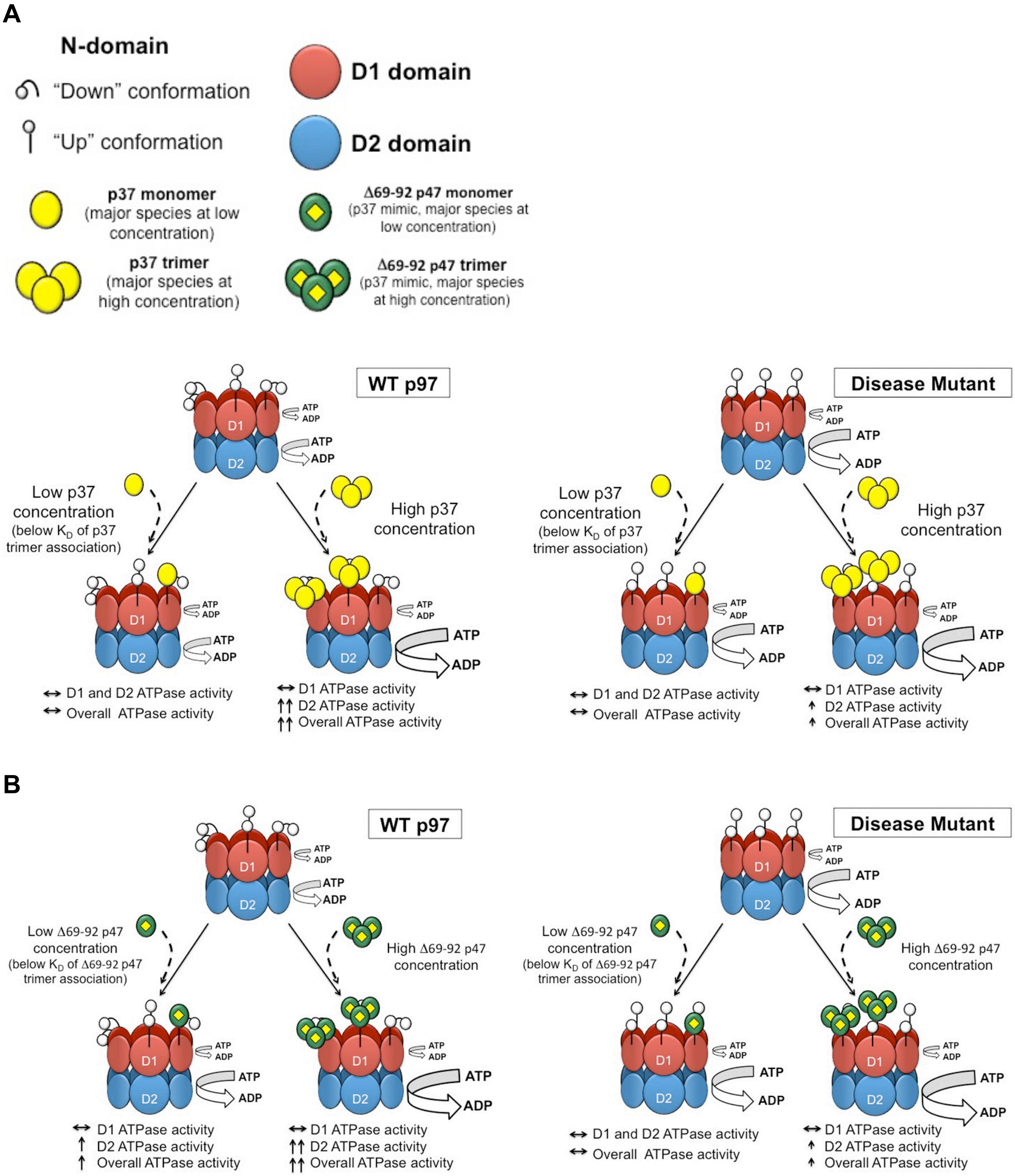

Figure S8. Cofactor-induced changes in p97 ATPase activity. Diagram showing the domain representation of human p97 and p37, or Δ69-92 p47 cofactors. Illustrations depict changes in p97 ATPase activity of WT and disease mutants caused by the presence of (A) p37, or (B) Δ69-92 p47.

SI References

1. Chou, T. F., Bulfer, S. L., Weihl, C. C., Li, K., Lis, L. G., Walters, M. A., Schoenen, F. J., Lin, H. J., Deshaies, R. J., and Arkin, M. R. (2014) Specific inhibition of p97/VCP ATPase and kinetic analysis demonstrate interaction between D1 and D2 ATPase domains. *J. Mol. Biol.* **426**, 2886-2899
2. Wang, Y., Satoh, A., Warren, G., and Meyer, H. H. (2004) VCIP135 acts as a deubiquitinating enzyme during p97-p47-mediated reassembly of mitotic Golgi fragments. *J. Cell Biol.* **164**, 973-978
3. Tang, D., Xiang, Y., and Wang, Y. (2010) Reconstitution of the cell cycle-regulated Golgi disassembly and reassembly in a cell-free system. *Nature protocols* **5**, 758-772
4. Fang, C. J., Gui, L., Zhang, X., Moen, D. R., Li, K., Frankowski, K. J., et al. (2015) Evaluating p97 Inhibitor Analogues for Their Domain Selectivity and Potency against the p97-p47 Complex. *ChemMedChem* **10**, 52-56